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Bacterial Preservation

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ABSTRACT:
The present work pertains to preservation of bacterial culture using liquid nutrient media incorporated with a simple cryoprotectant and stored at 4°C and -20°C for an observation period of one year without aid of any expensive equipments, chemicals, etc. A few combinations of different strengths of liquid culture media with varying proportions of cryoprotectant were employed for the maintenance of bacterial culture and observed for the viability of the organism. Certain percentage of regression was observed with the passage of time, however, appreciable number of viable organisms was observed even at twelfth month. It is therefore inferred that nutrient media, used with glycerine as cryoprotectant can sustain the viability of the organism with its characteristics intact for more than a year. Bacterial cultures developed in double concentration (D.C) Nutrient Broth (N.B) fortified with equal parts of 30% glycerine in single concentration (S.C) of Saline and in equal parts of N.B (D.C) with 40% glycerine in Saline (S.C) appeared to support viability of the organism upto 6 months when stored at 4° C ,well up twelve months at -20° C. Although culture developed in N.B.(S.C) 3 parts + One part of 30% glycerine in Saline(S.C) also supported well, but stood next in order of choice. *Staphylococcus aureus* was used as a model bacterial organism.The resultant ideal formulations developed is expected to be extendible to similar other organisms.

Key words: Bacterial preservation, Cryoprotectant, Survival percentage

INTRODUCTION:

Microbes though tiny are the source of many high value compounds that are useful to living things like humans, plants and animals. It is thus very important to preserve these useful strains for a long term for use in research and industrial applications [1]. Preservation by drying is known for thousands of years. Bacterial cells were suspended in melted nutrient gelatin containing ascorbic acid or sodium ascorbate in concentration of 0.25-0.5 %. Small quantities were dried over P_2O_5 at pressures of 100-300 mm. of mercury and stored in vacuuo over P_2O_5 at room temperature. A wide range of bacterial species of medical and veterinary importance was preserved by this method for 4 years [2].

Freezing is a good way of preservation of bacteria. Generally, the colder the storage temperature, the longer the culture are viable. Ice can damage cells by dehydration and increasing in salt concentration which can denature biomolecules, Ice can also rupture membranes. To lessen the negative effects of freezing, glycerol is often used as a cryoprotectant which keeps cells viable under freezing conditions.

[3]Morton and Pulaski recommended freezing the bacterial suspension immediately before and during simple drying in desiccators, and[4] Flosdorf and Mudd- freeze-drying for the preservation of biological products, including micro-organisms.

Spray drying is the most efficient dehydration technique for the preservation of microbial cultures at industrial scale since it can be carried out in a continuous mode [5]. However, one critical factor in spray drying processes is the high temperatures (85–90 °C) applied during the process, which can lead to heat and osmotic cellular stresses with deleterious impacts on sensitive microorganisms [6].

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The scalable foam-drying preservation technology under vacuum was brought by [7] Fu and Chen using skim milk.

The commonly used methods of preservation are (i) storage in liquid nitrogen,(ii) Freeze drying and (iii) Freezing at -70°C to -80°Cwith10% glycerol as a suitable cryoprotectant.
[8]Chavarri *et al* described that addition of 5% lactose or 5% sucrose or maltose as cryoprotective agents improve upon viability of *Streptococcus lactis*on freezing in liquid nitrogen (LN_2).

For spray drying and lyophilizationspecial equipments are required, and LN_2 vessels and the regular supply of LN_2 are the pre-requisite in these processes.

Storing cells in cryogenic freezers is the most effective and, as compared to laboratory freezers, ultra low freezers and freeze drying, the easiest method for long-term storage. The downside is cost and potential vulnerability of stocks to power outages, mechanical failures, and failed deliveries of liquid nitrogen. The operational and maintenance cost is expensive.

Cryopreservation is the most preferred technique for safeguarding microbial cultures without genetic or phenotypic alterations while maintaining cell viability in biological resource centers [9,10,11].
Freeze-drying, though an expensive technique for preserving microbial cells in industrial scale, it confers stability without culture transfers, retaining high cell viability after long-term storage [12].

Bacteria are usually maintained in the laboratory by regular sub culturing on solid nutrient, media at almost regular monthly intervals. By doing so the organisms are vulnerable to mutation at any point of time and therefore frequent sub culturing need to be avoided. This is also labour intensive, tedious, cost and space intensive while storing at 4°C.

Storage of culture, in liquid media at ultra low temperature is preferred for longer duration with cryoprotectant. However, the storage cabinet and its running cost of -80°C is expensive. Other equipments for -170°C or freeze drying is more expensive and labour intensive.

A need was felt for maintenance of culture in smaller laboratories for research purpose, in an affordable cost effective way without compromising with any loss of the original characteristics of the cultures. The present investigation was made for the maintenance of the bacterial cultures using various combinations of simple culture nutrient media with cryoprotectant in various proportions at 4°C and -20°C.

Staphylococcusaureus was used as a model bacterial organism. It's a non-spore forming, facultative anaerobic, non-motile, non-capsulated organism. The ideal formulations invented is expected to be extendible to similar other organisms also.

Most microbiologists prefer preservation of microorganisms at cryogenic temperatures (-80°C or -196°C) with 10-15% glycerol and/ or 5-10% DMSO, and there are few studies using other cryoprotectants.

In the present invention various percentage of glycerol with various concentration of bacteriological cultivation / growth media were tried with a bacteria for their preservation at 4°C and at -20°C. Their survival percentages were determined at monthly interval for the duration of twelve months.

MATERIALS AND METHODS:

Staphylococcusaureus was used as a model bacterial organism.

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The most widely used conventional media, used for growing the bacterial organism was nutrient broth and nutrient agar with the following recipe:

Nutrient Broth(N.B):
Beef extract powder (Bacto) 10 gm (1%)
Peptone powder (Rankem) 10 gm (1%)
Sodium chloride (Hi media) 5 gm (0.5%)
All media were sterilized by autoclaving at 15lb pressure (121°C for 30 minutes.

(i)Bacterial culture used (as a model): *Staphylococcus aureus,* isolated earlier from a patient and well characterized was available in the department of Biotechnology of VISM, Gwalior.
Before undertaking the work, selective agar medium like Nutrient agar and MacConkey agar were used to grow the organism, Gram staining was done. After phenotypic identification, further confirmation was done on the basis of its biochemical characterization.

Staphylococcus aureus
Growth on Nutrient agar: small, white creamy, circular, smooth colonies
On MacConkey agar: No growth

Coagulase	+
Catalase	+
Indole	-
M.R	+
VP	+
Nitrate reduction	+
Citrate utilization	+
Urease	+
Glucose	+
Lactose	+
Sucrose	+
Xylose	-

(ii) 0.85% Sodium chloride (Saline S.C-single concentration) as well as its double concentration (Saline D.C--double concentration) was prepared by dissolving in triple glass distilled water.

Distilled water to 1000 ml
pH 7.2 Nutrient Agar (N.A):
Beef extract powder (Bacto) 10 gm (1%)
Peptone powder (Rankem) 10 gm (1%)
Sodium chloride (Hi media) 5 gm (0.5%)
Distilled water to 1000 ml
pH 7.2
Agar-Agar 20gm (2%)

Saline (S.C-Single concentration)
Sodium chloride 8.5 gm
Distilled water to 1000 ml
Saline (D.C-Double concentration)
Sodium chloride 17.0 gm
Distilled water to 1000 ml
(iii)Conventional Nutrient broth (N.B -S.C) was prepared with its ingredients as above.
(iv) SimilarlyNutrient broth (D.C) was prepared with above ingredients in double concentration (as follows).

Beef extract powder (Bacto) 20 gm
Peptone powder Rankem) 20 gm
Sodium chloride (Hi meduia) 10 gm
Distilled water to 1000 ml
pH 7.2

All preparations (Saline and media) were sterilized by autoclaving at 15lb pressure (121°C) for 30minutes.
(v)Cryoprotectant**Glycerine** was solubilized **inSaline** as well as in **nutrient broth** as follows:

(a) 30% glycerine in Saline (S.C)
Glycerine (Rankem) 30 ml
Saline (S.C) 100ml
(b) 30% glycerine in Nutrient broth(S.C)
Glycerine (Rankem) 30 ml
Nutrient broth (S.C) 100ml
(c) 30% glycerine in nutrient broth (D.C)
Glycerine (Rankem) 30 ml
Nutrient broth (D.C) 100ml
(d) 40% glycerine in Saline (S.C)
Glycerine (Rankem) 40 ml
Saline (S.C) 100ml
(e) 40% glycerine in Nutrient broth(S.C)
Glycerine (Rankem) 40 ml
Nutrient broth (S.C) 100ml

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(f) 40% glycerine in Nutrient broth(D.C)
Glycerine (Rankem) 40 ml
Nutrient broth (D.C) 100ml
(g) 60% glycerine in Saline (S.C)
Glycerine (Rankem) 60 ml
Nutrient broth (S.C) 100ml
(h) 80% glycerine in Saline (S.C)
Glycerine (Rankem) 80 ml
Nutrient broth (S.C) 100ml

These preparations were sterilized by autoclaving at 10 lb pressure for 30 minutes. *Staphylococcus aureus* were grown overnight at 37^{0}C in nutrient broth (NB) of single concentration (S.C) as well as in NB of double concentration (D.C).

(vi) The various **combinations** of **Cryoprotectant (Glycerine)** and Bacterial growth in **nutrient media** were prepared as follows:
(a) Bacterial culture in N.B (S.C)-1part + 30% glycerine in Saline (S.C)-1part
(b) Bacterial culture in N.B (S.C)-1part + 30% glycerine in N.B (S.C)-1part
(c) Bacterial culture in N.B (S.C)-1part + 30% glycerine in N.B (D.C)-1part
(d) Bacterial culture in N.B (S.C)-1part + 40% glycerine in Saline (S.C)-1part
(e) Bacterial culture in N.B (S.C)-1part + 40% glycerine in N.B (S.C)-1part
(f) Bacterial culture in N.B (S.C)-1part + 40% glycerine in N.B (D.C)-1part

(i) Each preparation was aliquoted into four and two of them were stored at 4^{0}Cand the other half at -20^{0}C.

(ii)On 0 day i.e. on the day of preparation of the above combinations, each preparation was diluted in small volume in normal saline as 10^{-1} through 10^{-5}. Each dilution was surface plated on nutrient agar in a 4 inch dia. Petri dish with 1ml of the diluted material in duplicate, excess fluid was drawn off and incubated overnight at 37^0 C.

(g) Bacterial culture in N.B (D.C)-1part + 30% glycerine in Saline (S.C)-1part
(h) Bacterial culture in N.B (D.C)-1part + 30% glycerine in N.B (S.C)-1part
(i) Bacterial culture in N.B (D.C)-1part + 30% glycerine in N.B (D.C)-1part
(j) Bacterial culture in N.B (D.C)-1part + 40% glycerine in Saline (S.C)-1part
(k) Bacterial culture in N.B (D.C)-1part + 40% glycerine in N.B (S.C)-1part
(l) Bacterial culture in N.B (D.C)-1part + 40% glycerine in N.B (D.C)-1part
(m) Bacterial culture in N.B (S.C)-1part + 30% glycerine in Saline (D.C)-1part
(n) Bacterial culture in N.B (D.C)-1part + 40% glycerine in Saline (D.C)-1part
(o) Bacterial culture in N.B (S.C)-1part + 40% glycerine in Saline (D.C)-1part
(p) Bacterial culture in N.B (S.C)-1part + 60% glycerine in Saline (S.C)-1part
(q) Bacterial culture in N.B (S.C)-1part + 80% glycerine in Saline (S.C)-1part
(r) Bacterial culture in N.B (S.C)-1part + 30% glycerine in Saline (S.C)-2parts
(s) Bacterial culture in N.B (S.C)-2parts+ 30% glycerine in Saline (S.C)-1part
(t) Bacterial culture in N.B (S.C)-1part + 30% glycerine in Saline (S.C)-3parts
(u) Bacterial culture in N.B (S.C)-3parts + 30% glycerine in Saline (S.C)-1part
(v) **Control group**: Bacterial culture in N.B (S.C)-1part + Saline (S.C)-1part

METHODOLOGY:
(iii) On the following day colony counts were taken from each plate and an average of the two was made. After preliminary screening heavy growth was noted with 10^{-1} and10^{-2} dilutions, and therefore counts were taken through 10^{-3} to 10^{-5} dilutionssubsequently. Since the optimum dilution 10^{-4} gave considerable counts in almost all, this dilution was considered throughout the study. Wherever the count was more in 10^{-4} dilution, count at 10^{-5} was taken and multiplied by the dilution factor 10 so as to derive at the absolute count at 10^{-4} dilution.

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(iv) The study period was of twelve months duration, performing the survival study of the organism every month, however, in the first month study was done on 12th day.

(v) Control was kept alongside without any chryoprotectant.

(vi) The preserved materials underwent the situation of electricity failure a couple of times during the study period.

(vii)Sample of materials were taken from the same stock, stored at 4^0C as well as at -20^0C, after thawing, throughout the study.

(viii) The survived organisms were checked for the preservation of their characteristics, as above, every month while determining their survival percentage.

(ix)The surface viable count method described by [13]Miles and Misra, was followed with slight modifications, under careful standardized conditions. When adequate number of colonies were counted this gave results with a high degree of precision.

RESULTS:

(i)*Staphylococcus aureus,* when grown in (a) nutrient broth (Double concentration) and mixed with 30% glycerine (solubilized in single concentration of saline) in equal volumes and preserved at 4^0C, it supported the survival of the organism 99% on 12th day, and there was a gradual regression to 0.4% on 7th month. This combination appears to the best over (b) culture in N.B (D.C)-1part+ 40% glycerine in saline (S.C)-1part,while (c) culture in N.B (S.C)-1part+ 30% glycerine in N.B(S.C)-1part supported upto 6 months only.

(ii) Simultaneously, when the organisms were grown in (a) nutrient broth (Double concentration) and mixed with 30% glycerine (solubilized in single concentration of saline) in equal volumes and preserved at at -20^0C, it supported the survival of the organism 99% on 12th day, and there was a gradual regression to 28 % on 12 th month. This combination, however, appears to the best over (b) culture in N.B (S.C)-3parts + 30% glycerine in N.B(S.C)-1part supported 12% on 12th month and (c) culture in N.B (D.C)-1part+ 40% glycerine in saline (S.C)-1part with 10% upto 12 months.

Data are presented in **Tables** (1 to 20) as well as depicted graphically in **figures**(1 to 20).**Each set of data** is presented both **in tabular form** as well as **in graphical form (Fig.). Their counterpart in percentage** is also presented **in tabular form** as well as **in graphical form (Fig.)**.

For an example Table 1 shows Bacterial preservation at 4°C (with digital- Survival number x 10^4) and Fig 1.shows Bacterial preservation at 4°C graphically (with Survival number x 10^4); Table 2.Shows Bacterial preservation at 4°C (with digital Survival percentage) and Fig 2. Shows Bacterial preservation at 4°C graphically (with Survival percentage**).**

Similarly other tables and figures represent Survival number in digital form and in percentage graphically at 4°C as well as at -20°C.

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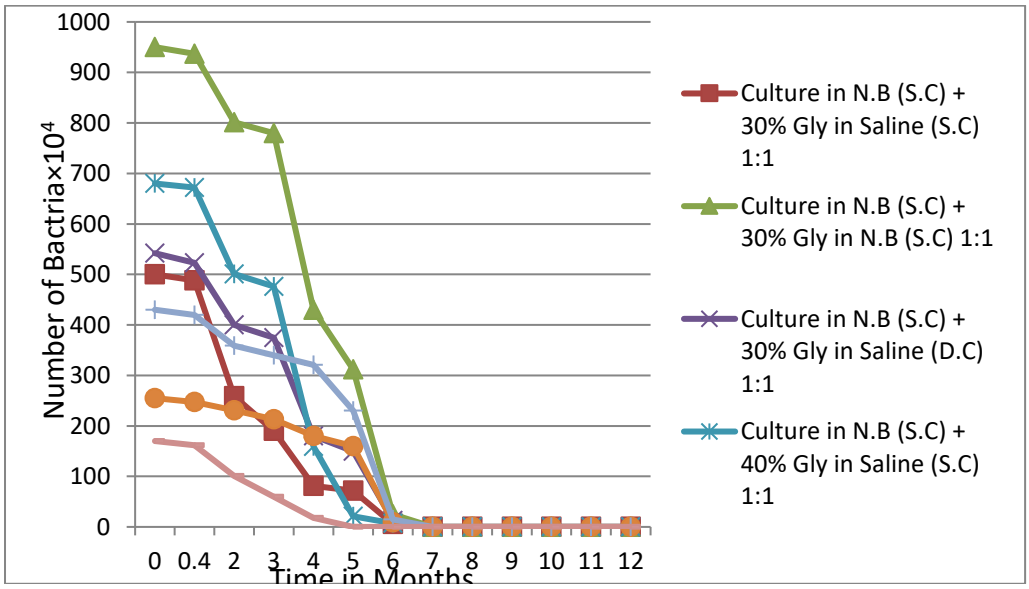
Table 1.**Bacterial preservation at 4°C (Survival number x 10^4)**

Time (Months)	Culture in N.B (S.C) + 30% Gly in Saline (S.C) 1:1	Culture in N.B (S.C) + 30% Gly in N.B (S.C) 1:1	Culture in N.B (S.C) + 30% Gly in Saline (D.C) 1:1	Culture in N.B (S.C) + 40% Gly in Saline (S.C) 1:1	Culture in N.B (S.C) + 40% Gly in N.B (S.C) 1:1	Culture in N.B (S.C) + 40% Gly in N.B (D.C) 1:1	Control Culture in N.B (S.C) + Saline (S.C) 1:1
0 Day	500	950	542	680	255	430	170
0.4	488	937	523	672	247	420	162
2	259	801	400	501	231	359	101
3	190	780	375	476	213	340	60
4	81	430	180	159	180	321	18
5	72	312	150	21	160	230	0
6	6	25	13	7	9	15	0
7 to 12	0	0	0	0	0	0	0

NB;Nutrient Broth :S.C: Single Concentration; D.C:Double Concentration: Gly.: Glycerine



NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

Fig 1.**Bacterial preservation at 4°C (Survival number x 10^4)**

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Table 2.**Bacterial preservation at 4°C (Survival percentage)**

Time (Months)	Culture in N.B (S.C) + 30% Gly in Saline (S.C) 1:1	Culture in N.B (S.C) + 30% Gly in N.B (S.C) 1:1	Culture in N.B (S.C) + 30% Gly in Saline (D.C) 1:1	Culture in N.B (S.C) + 40% Gly in Saline (S.C) 1:1	Culture in N.B (S.C) + 40% Gly in N.B (S.C) 1:1	Culture in N.B (S.C) + 40% Gly in N.B (D.C) 1:1	Control Culture in N.B (S.C) + Saline (S.C) 1:1
0 Day	100	100	100	100	100	100	100
0.4	97.6	98.63	96.49	98.82	96.86	97.67	95.29
2	51.8	84.31	73.8	73.67	90.58	83.48	59.41
3	38	82.1	69.18	70	83.52	79.06	35.29
4	16.2	45.26	33.21	23.38	70.58	74.65	10.58
5	14.4	32.84	27.67	3.08	62.74	53.48	0
6	1.2	2.63	2.39	1.02	3.52	3.48	0
7 to 12	0	0	0	0	0	0	0

NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

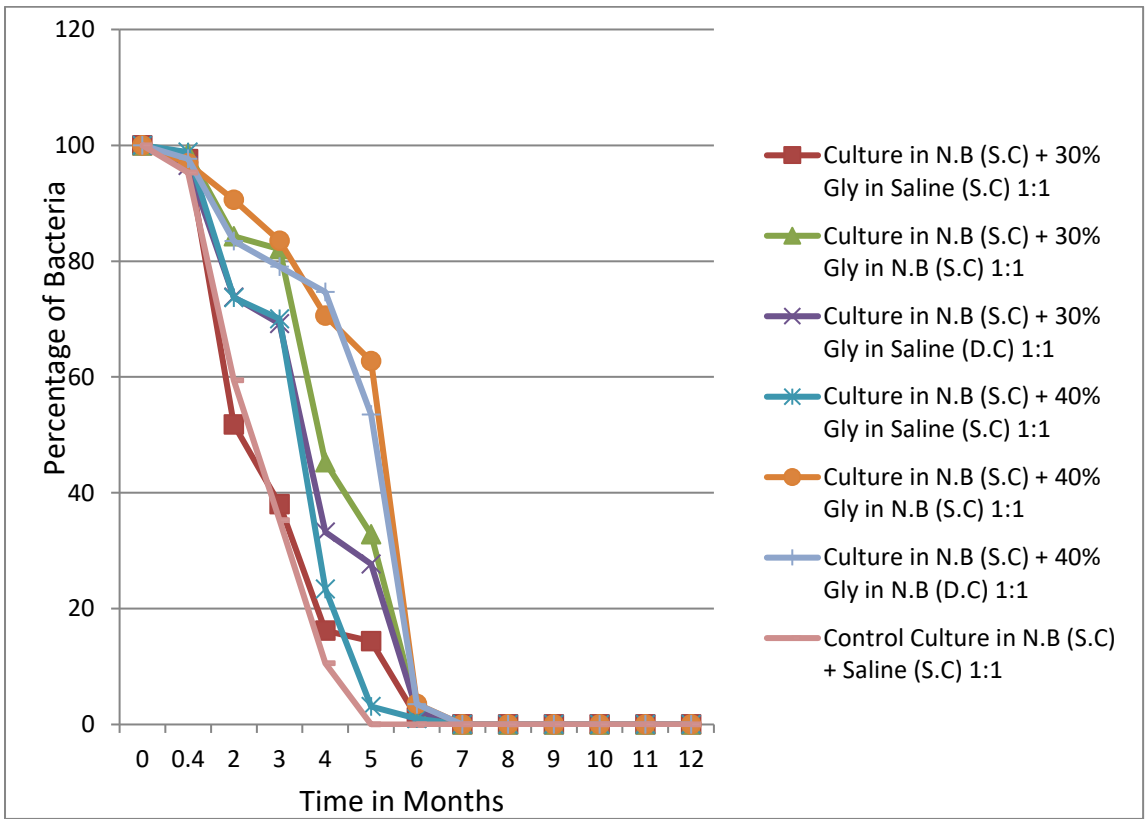

Fig 2.**Bacterial preservation at 4°C (Survival percentage)**

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Table 3.**Bacterial preservation at -20°C (Survival number x 10^4)**

Time (Months)	Culture in N.B (S.C) + 30% Gly in Saline (S.C) 1:1	Culture in N.B (S.C) + 30% Gly in N.B (S.C) 1:1	Culture in N.B (S.C) + 30% Gly in Saline (D.C) 1:1	Culture in N.B (S.C) + 40% Gly in Saline (S.C) 1:1	Culture in N.B (S.C) + 40% Gly in N.B (S.C) 1:1	Culture in N.B (S.C) + 40% Gly in N.B (D.C) 1:1	Control Culture in N.B (S.C) + Saline (S.C) 1:1
0 Day	1035	638	639	192	282	396	382
0.4	1027	633	635	187	276	390	375
2	402	337	225	180	147	232	150
3	323	301	177	176	101	205	60
4	221	285	122	167	93	179	52
5	150	260	88	162	80	146	47
6	50	147	84	154	73	90	40
7	0	50	82	151	67	58	21
8	0	41	76	100	65	58	15
9	0	12	71	60	60	50	0
10	0	0	60	54	52	53	0
11	0	0	14	11	52	27	0
12	0	0	10	0	40	17	0

NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

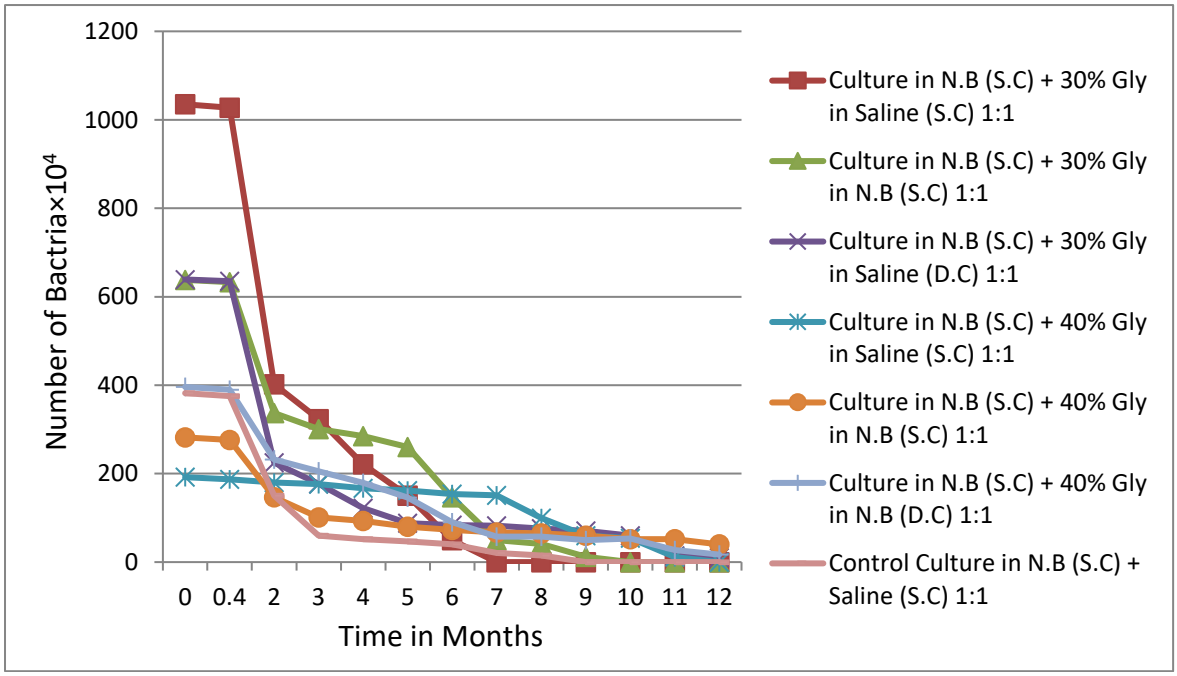

Fig 3.**Bacterial preservation at -20°C (Survival number x 10^4)**
NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

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Table 4.**Bacterial preservation at -20°C (Survival percentage)**

Time (Months)	Culture in N.B (S.C) + 30% Gly in Saline (S.C) 1:1	Culture in N.B (S.C) + 30% Gly in N.B (S.C) 1:1	Culture in N.B (S.C) + 30% Gly in Saline (D.C) 1:1	Culture in N.B (S.C) + 40% Gly in Saline (S.C) 1:1	Culture in N.B (S.C) + 40% Gly in N.B (S.C) 1:1	Culture in N.B (S.C) + 40% Gly in N.B (D.C) 1:1	Control Culture in N.B (S.C) + Saline (S.C) 1:1
0 Day	100	100	100	100	100	100	100
0.4	99.22	99.21	99.37	97.39	97.87	98.48	98.16
2	38.84	52.82	35.21	93.75	52.12	58.58	39.26
3	31.2	47.17	27.69	91.66	35.81	51.76	15.7
4	21.35	44.67	19.09	86.97	32.97	45.2	13.61
5	14.49	40.75	13.77	84.37	28.36	36.86	12.3
6	4.83	23.22	13.14	80.2	25.88	22.72	10.47
7	0	7.83	12.83	78.64	23.75	14.64	5.49
8	0	6.42	11.89	52.08	23.04	14.64	3.92
9	0	1.89	11.11	31.25	21.27	12.62	0
10	0	0	9.38	28.12	18.43	13.38	0
11	0	0	2.19	5.72	18.43	6.18	0
12	0	0	1.56	0	14.18	4.29	0

NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

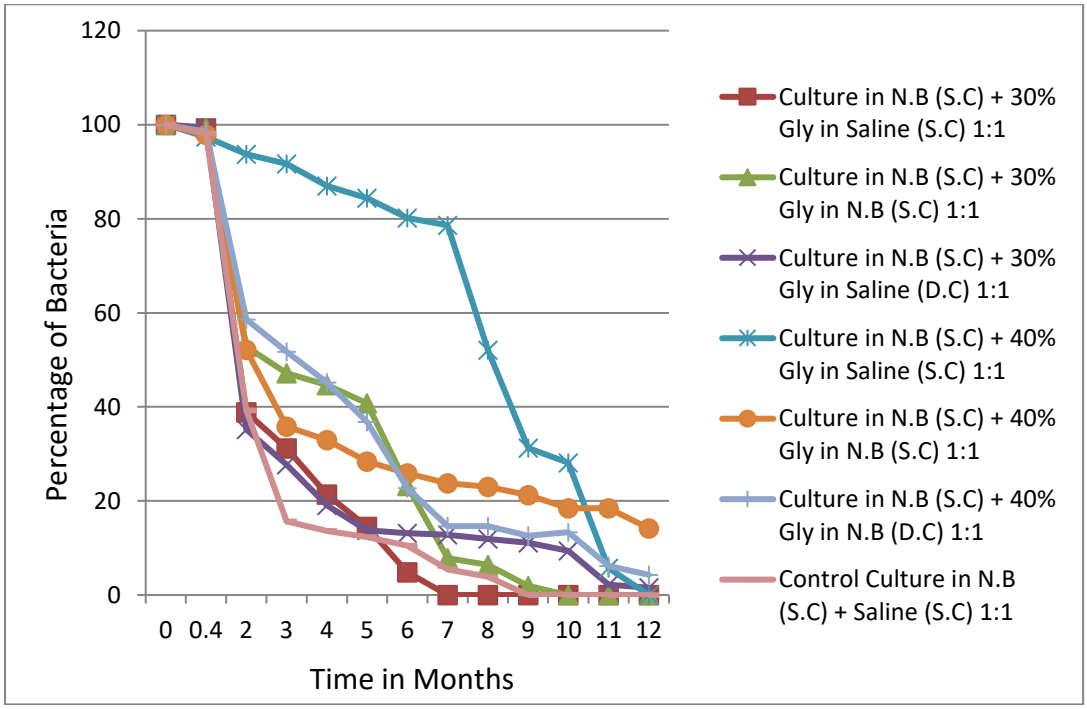

Fig 4.**Bacterial preservation at -20°C (Survival percentage)**
NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

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Table 5.**Bacterial preservation at 4°C (Survival number x 10^4)**

Time (Months)	Culture in N.B (D.C) + 30% Gly in Saline (S.C) 1:1	Culture in N.B (D.C) + 30% Gly in N.B (S.C) 1:1	Culture in N.B (D.C) + 30% Gly in Saline (D.C) 1:1	Culture in N.B (D.C) + 40% Gly in Saline (S.C) 1:1	Culture in N.B (D.C) + 40% Gly in N.B (S.C) 1:1	Culture in N.B (D.C) + 40% Gly in N.B (D.C) 1:1	Control Culture in N.B (S.C) + Saline (S.C) 1:1
0 Day	3670	167	120	2990	476	491	170
0.4	3660	162	113	2982	468	480	162
2	1174	120	93	1474	279	173	101
3	474	88	62	400	182	48	60
4	450	12	50	391	121	30	18
5	430	3	42	342	32	23	0
6	219	1	21	101	11	14	0
7	15	0	6	7	0	8	0
8 to 12	0	0	0	0	0	0	0

NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

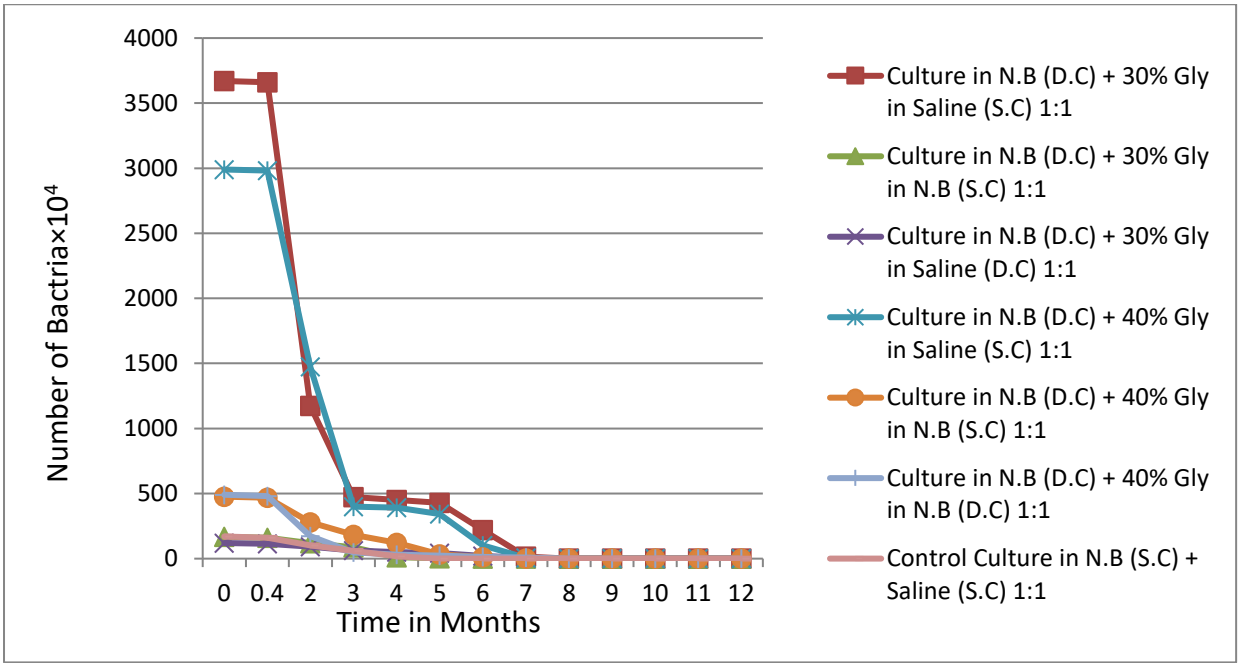

Fig 5.**Bacterial preservation at 4°C (Survival number x 10^4)**
NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

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Table 6.**Bacterial preservation at 4°C (Survival percentage)**

Time (Months)	Culture in N.B (D.C) + 30% Gly in Saline (S.C) 1:1	Culture in N.B (D.C) + 30% Gly in N.B (S.C) 1:1	Culture in N.B (D.C) + 30% Gly in Saline (D.C) 1:1	Culture in N.B (D.C) + 40% Gly in Saline (S.C) 1:1	Culture in N.B (D.C) + 40% Gly in N.B (S.C) 1:1	Culture in N.B (D.C) + 40% Gly in N.B (D.C) 1:1	Control Culture in N.B (S.C) + Saline (S.C) 1:1
0 Day	100	100	100	100	100	100	100
0.4	99.72	97	94.16	99.73	93.31	97.75	95.29
2	31.98	71.85	77.5	49.29	58.61	35.23	59.41
3	12.91	52.69	51.66	13.37	38.23	9.77	35.29
4	12.26	7.18	41.66	13.07	25.42	6.1	10.58
5	11.71	1.79	35	11.43	6.72	4.68	0
6	5.96	0.59	17.5	3.37	2.31	2.858	0
7	0.4	0	5	0.23	0	1.62	0
8 to 12	0	0	0	0	0	0	0

NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

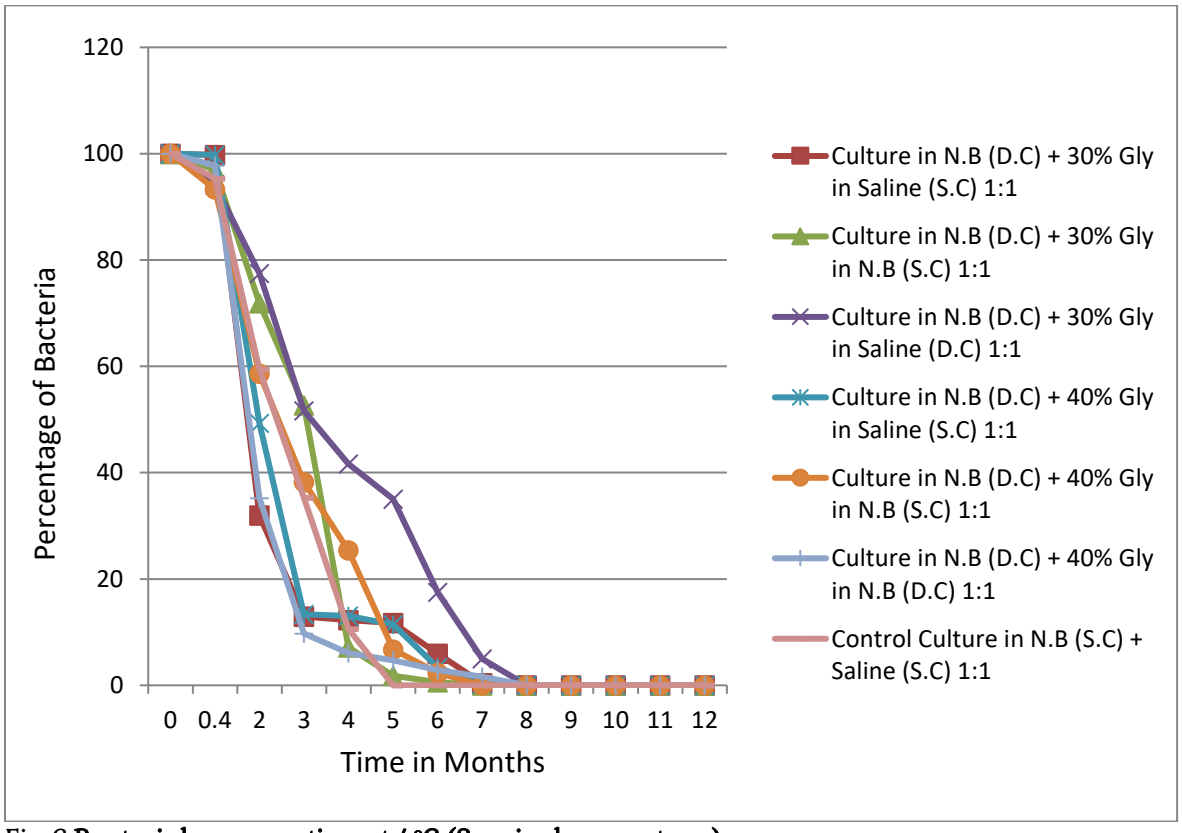

Fig 6.**Bacterial preservation at 4°C (Survival percentage)**
NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly. : Glycerine

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Table 7.**Bacterial preservation at -20°C (Survival number x 10⁴)**

Time (Months)	Culture in N.B (D.C) + 30% Gly in Saline (S.C) 1:1	Culture in N.B (D.C) + 30% Gly in N.B (S.C) 1:1	Culture in N.B (D.C) + 30% Gly in Saline (D.C) 1:1	Culture in N.B (D.C) + 40% Gly in Saline (S.C) 1:1	Culture in N.B (D.C) + 40% Gly in N.B (S.C) 1:1	Culture in N.B (D.C) + 40% Gly in N.B (D.C) 1:1	Control Culture in N.B (S.C) + Saline (S.C) 1:1
0 Day	1421	1326	847	2290	971	438	382
0.4	1416	1321	842	2287	960	432	375
2	1203	1100	750	1500	750	322	150
3	860	627	528	400	620	212	60
4	724	602	459	339	553	201	52
5	650	550	320	271	459	200	47
6	603	505	256	262	410	192	40
7	529	400	150	291	350	180	21
8	510	368	131	357	310	155	15
9	491	336	129	280	233	149	0
10	490	325	122	275	220	142	0
11	402	227	121	263	181	134	0
12	400	210	115	245	160	115	0

NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

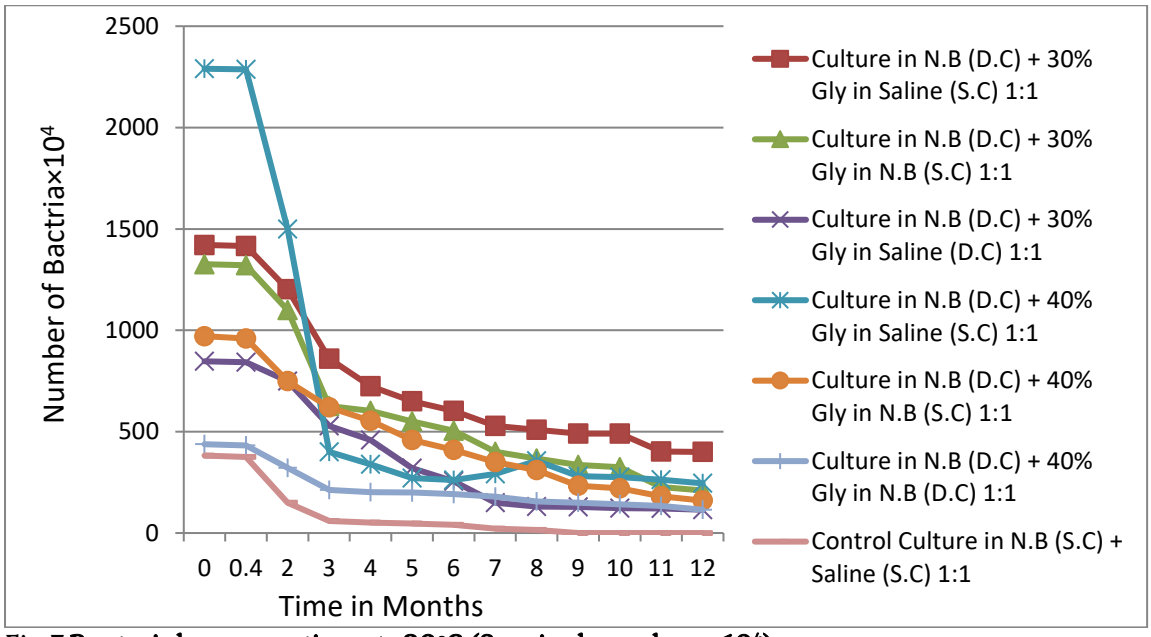

Fig 7.**Bacterial preservation at -20°C (Survival number x 10⁴)**
NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

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Table 8.**Bacterial preservation at -20°C (Survival percentage)**

Time (Months)	Culture in N.B (D.C) + 30% Gly in Saline (S.C) 1:1	Culture in N.B (D.C) + 30% Gly in N.B (S.C) 1:1	Culture in N.B (D.C) + 30% Gly in Saline (D.C) 1:1	Culture in N.B (D.C) + 40% Gly in Saline (S.C) 1:1	Cultre in N.B (D.C) + 40% Gly in N.B (S.C) 1:1	Culture in N.B (D.C) + 40% Gly in N.B (D.C) 1:1	Control Culture in N.B (S.C) + Saline (S.C) 1:1
0 Day	100	100	100	100	100	100	100
0.4	99.64	99.62	99.4	99.86	98.86	98.63	98.16
2	84.65	82.95	88.54	65.5	77.23	73.51	39.26
3	60.52	47.28	62.33	17.46	63.85	48.4	15.7
4	50.95	45.39	54.19	14.8	56.95	45.89	13.61
5	45.74	41.47	37.78	13.14	47.27	45.66	12.3
6	42.43	38.08	30.22	13.1	42.22	43.83	10.47
7	37.22	30.16	17.7	12.7	36.04	41.09	5.49
8	35.89	27.75	15.46	12.62	31.92	35.38	3.92
9	34.55	25.33	15.23	12.22	23.99	34.01	0
10	34.48	24.5	14.4	12	22.65	32.42	0
11	28.28	17.11	14.28	11.48	18.64	30.59	0
12	28.14	15.83	13.37	10.69	16.47	26.25	0

NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

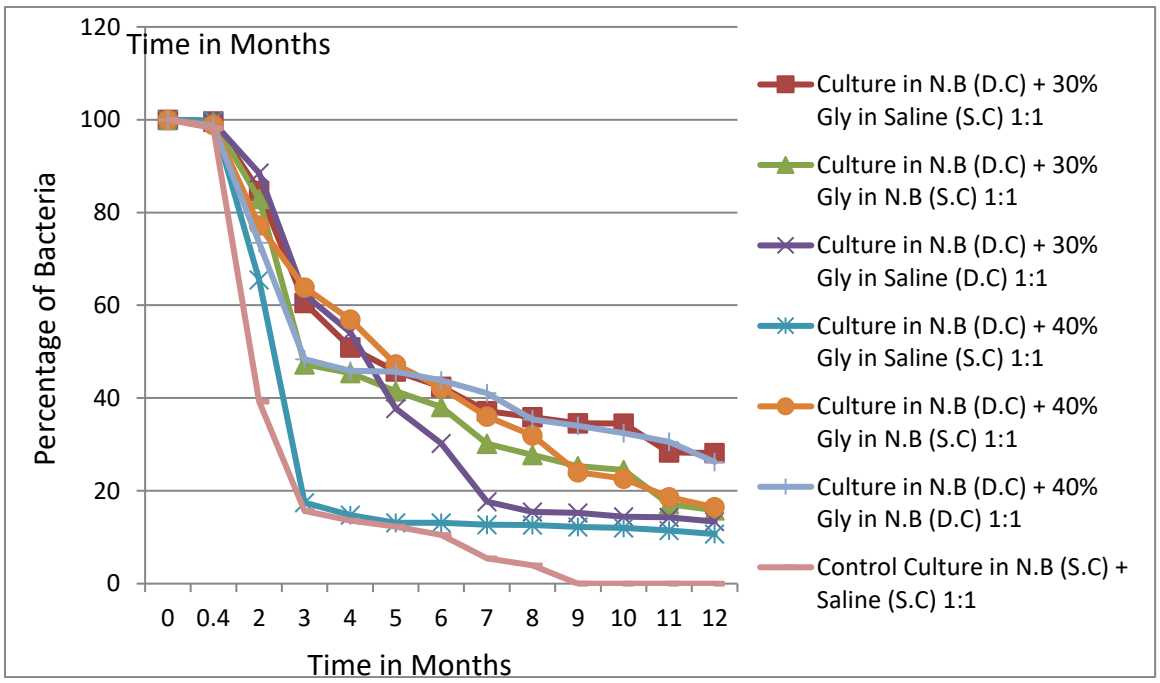

Fig 8.**Bacterial preservation at -20°C (Survival percentage)**
NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

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Table 9.**Bacterial preservation at 4°C (Survival number x 10^4)**

Time (Months)	Culture in N.B (S.C) + 30% Gly in Saline (D.C) 1:1	Culture in N.B (D.C) + 40% Gly in saline (D.C) 1:1	Culture in N.B (S.C) + 40% Gly in Saline (D.C) 1:1	Culture in N.B (S.C) + 60% Gly in Saline (S.C) 1:1	Culture in N.B (S.C) + 80% Gly in Saline (S.C) 1:1	Control Culture in N.B (S.C) + Saline (S.C) 1:1
0 Day	291	312	126	162	35	170
0.4	284	302	122	156	31	162
2	184	179	101	96	10	101
3	86	67	80	42	0	60
4	70	51	72	33	0	18
5	22	10	15	21	0	0
6	6	0	2	2	0	0
7 to 12	0	0	0	0		0

NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

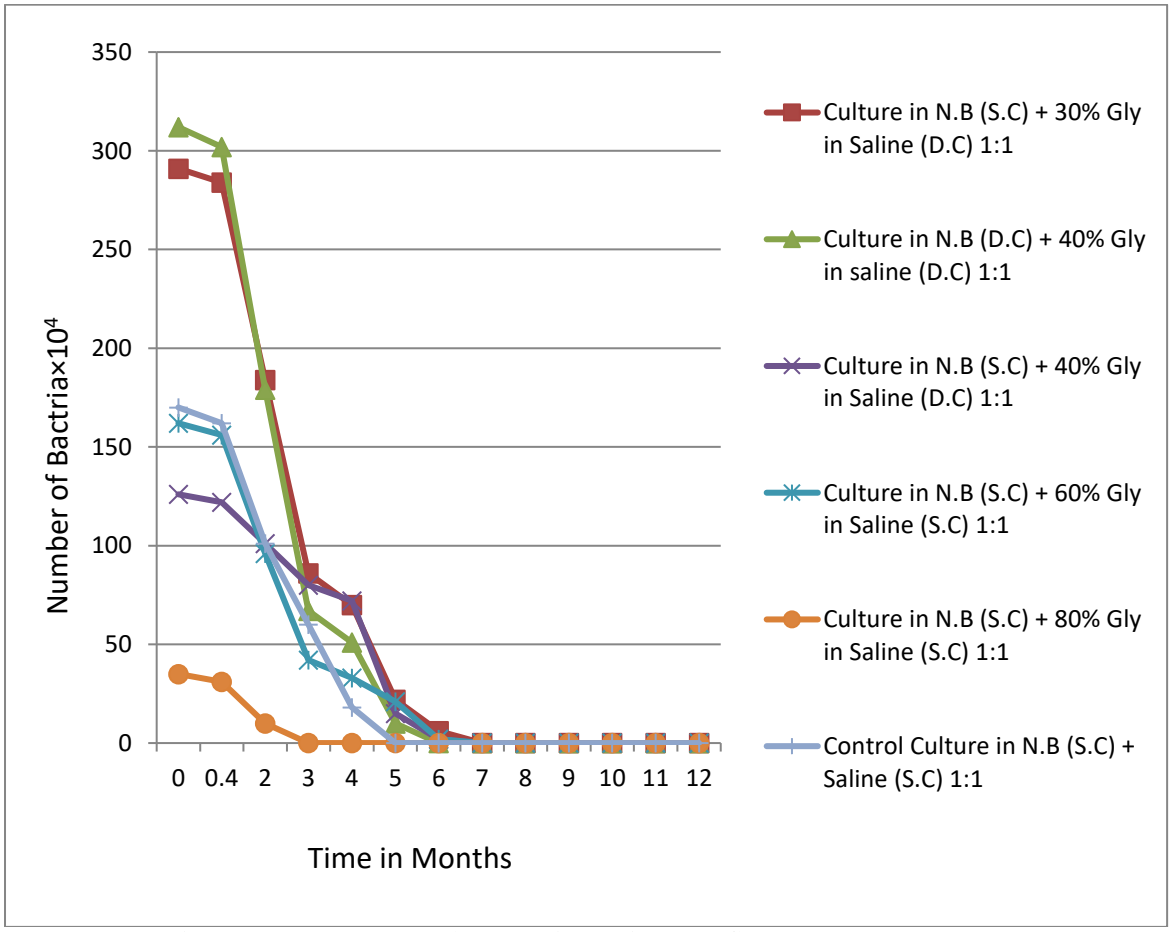

Fig 9.**Bacterial preservation at 4°C (Survival number x 10^4)**
NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

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Table 10.**Bacterial preservation at 4°C (Survival percentage)**

Time (Months)	Culture in N.B (S.C) + 30% Gly in Saline (D.C) 1:1	Culture in N.B (D.C) + 40% Gly in saline (D.C) 1:1	Culture in N.B (S.C) + 40% Gly in Saline (D.C) 1:1	Culture in N.B (S.C) + 60% Gly in Saline (S.C) 1:1	Culture in N.B (S.C) + 80% Gly in Saline (S.C) 1:1	Control Culture in N.B (S.C) + Saline (S.C) 1:1
0	100	100	100	100	100	100
0.4	97.59	96.79	96.82	96.29	88.57	95.29
2	63.23	57.37	80.15	59.25	28.57	59.41
3	29.55	21.47	63.49	25.92	0	35.29
4	24.05	16.34	57.14	20.37	0	10.58
5	7.56	3.2	11.9	12.96	0	0
6	2.06	0	1.58	1.23	0	0
7 to 12	0	0	0	0	0	0

NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

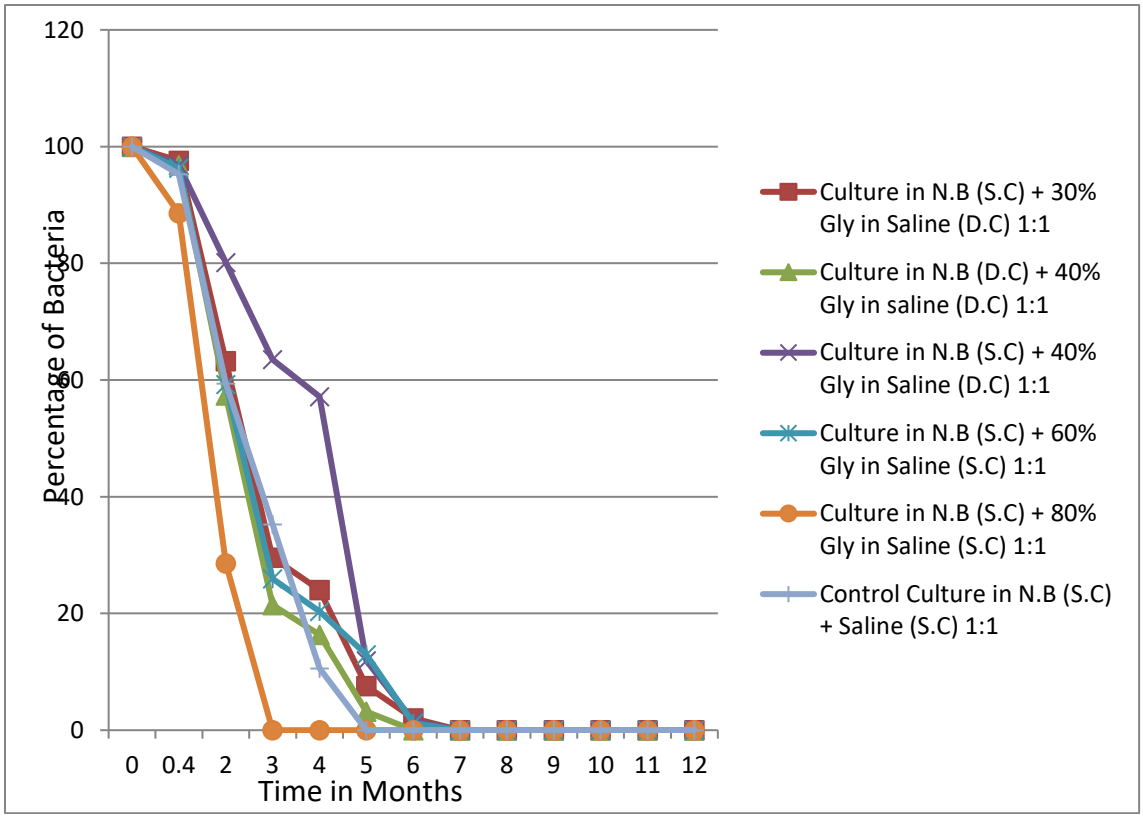

Fig 10.**Bacterial preservation at 4°C (Survival percentage)**

NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

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Table 11.**Bacterial preservation at -20°C (Survival number x 10^4)**

Time (Months)	Culture in N.B (S.C) + 30% Gly in Saline (D.C) 1:1	Culture in N.B (D.C) + 40% Gly in saline (D.C) 1:1	Culture in N.B (S.C) + 40% Gly in Saline (D.C) 1:1	Culture in N.B (S.C) + 60% Gly in Saline (S.C) 1:1	Culture in N.B (S.C) + 80% Gly in Saline (S.C) 1:1	Control Culture in N.B (S.C) + Saline (S.C) 1:1
0 Day	1078	1174	1150	1429	912	382
0.4	1070	1168	1143	1125	900	375
2	850	830	740	1021	819	150
3	740	700	662	981	705	60
4	459	525	520	749	525	52
5	319	321	300	424	322	47
6	209	150	250	234	151	40
7	182	140	200	201	143	32
8	175	131	160	150	140	15
9	136	121	110	100	120	0
10	122	112	105	85	75	0
11	99	100	90	81	62	0
12	90	85	75	70	52	0

NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

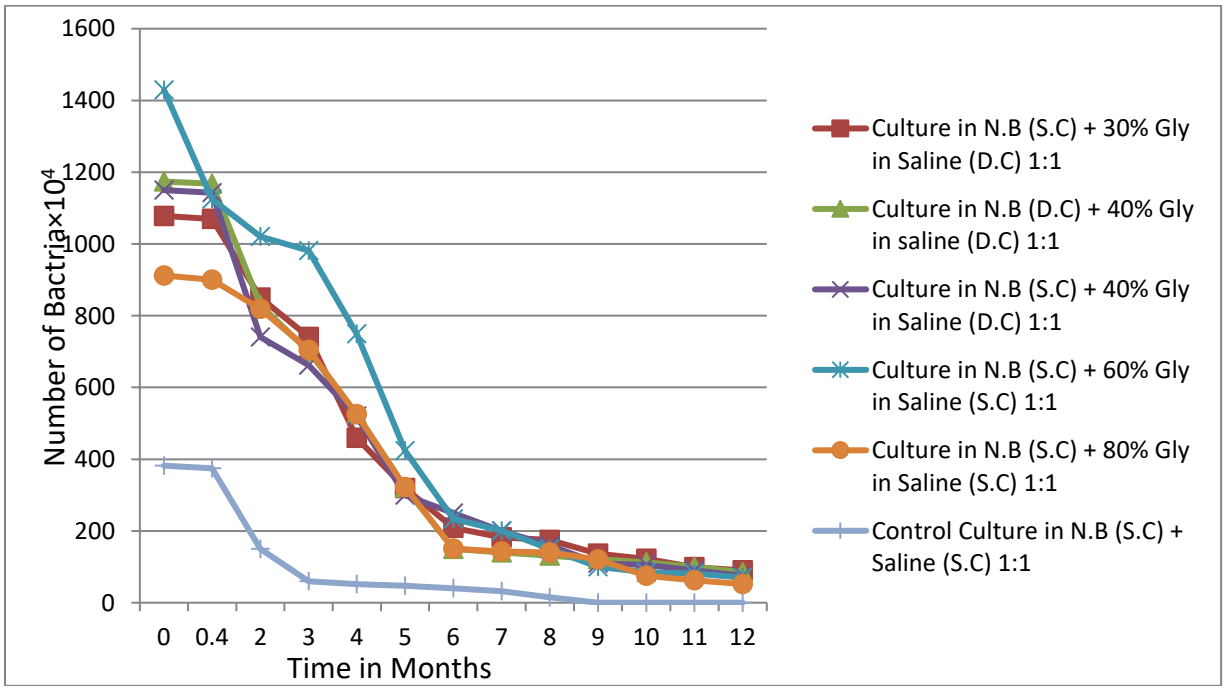

Fig. 11.**Bacterial preservation at -20°C (Survival number x 10^4)**
NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

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Table 12.**Bacterial preservation at -20°C (Survival percentage)**

Time (Months)	Culture in N.B (S.C) + 30% Gly in Saline (D.C) 1:1	Culture in N.B (D.C) + 40% Gly in saline (D.C) 1:1	Culture in N.B (S.C) + 40% Gly in Saline (D.C) 1:1	Culture in N.B (S.C) + 60% Gly in Saline (S.C) 1:1	Culture in N.B (S.C) + 80% Gly in Saline (S.C) 1:1	Control Culture in N.B (S.C) + Saline (S.C) 1:1
0 Day	100	100	100	100	100	100
0.4	99.25	99.48	99.39	99.64	98.68	98.16
2	78.84	70.69	64.34	90.43	89.8	39.26
3	68.64	59.62	57.569	86.89	77.3	15.7
4	42.59	44.71	45.1	66.34	57.56	13.61
5	29.59	27.34	26.08	37.55	35.3	12.3
6	19.38	12.77	21.73	20.72	16.55	10.47
7	16.88	11.92	17.39	17.8	15.67	8.37
8	16.23	11.15	13.91	13.28	15.35	3.92
9	12.61	10.3	9.56	8.85	13.15	0
10	11.31	9.54	9.13	7.52	8.22	0
11	9.18	8.51	7.82	7.17	6.79	0
12	8.34	7.24	6.52	6.2	5.7	0

NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

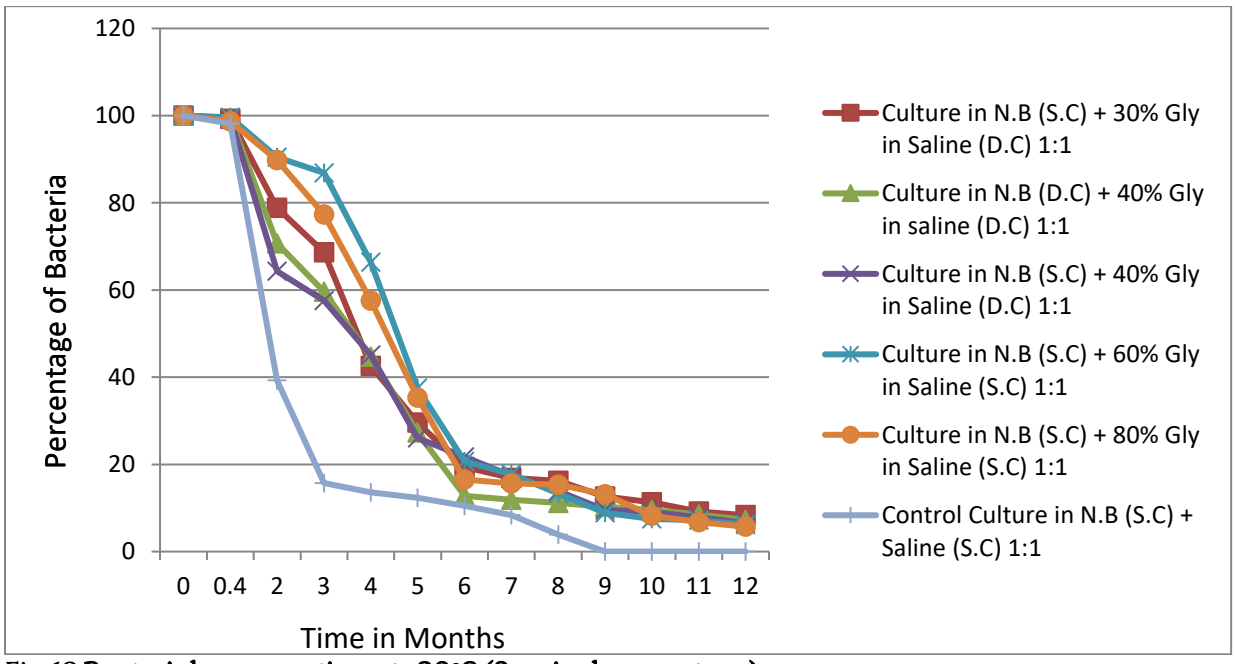

Fig 12.**Bacterial preservation at -20°C (Survival percentage)**

NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

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Table 13.**Bacterial preservation at 4°C (Survival number x 10^4)**

Time (Months)	Culture in N.B (S.C) + 30% Gly in Saline (S.C) 1:2	Culture in N.B (S.C) + 30% Gly in saline (S.C) 2:1	Culture in N.B (S.C) + 30% Gly in Saline (S.C) 1:3	Culture in N.B (S.C) + 30% Gly in Saline (S.C) 3:1	Control Culture in N.B (S.C) + Saline (S.C) 1:1
0 Day	366	632	148	81	170
0.4	360	627	143	75	162
2	202	428	75	27	101
3	127	83	30	7	60
4	101	60	25	3	18
5	52	22	12	2	0
6	14	0	0	0	0
7 to 12	0	0	0	0	0

NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

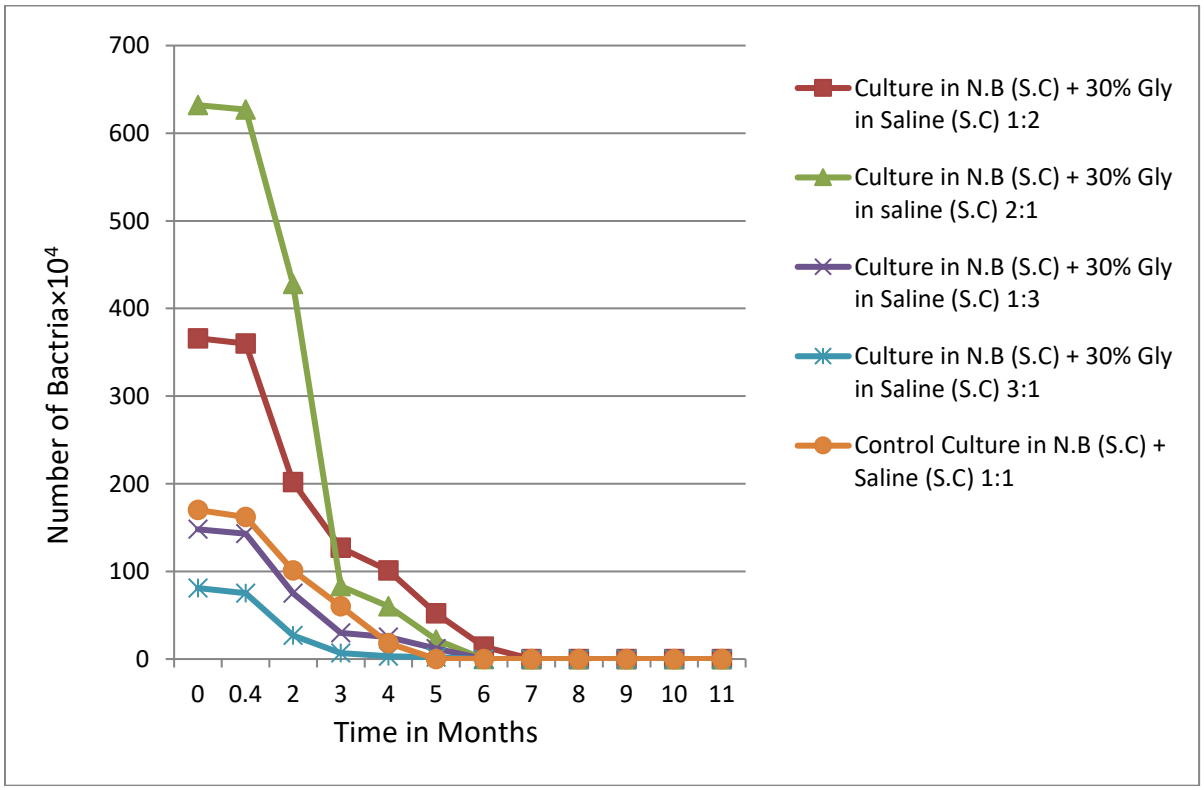

Fig 13.**Bacterial preservation at 4°C (Survival number x 10^4)**
NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

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Table 14.**Bacterial preservation at 4°C (Survival percentage)**

Time (Months)	Culture in N.B (S.C) + 30% Gly in Saline (S.C) 1:2	Culture in N.B (S.C) + 30% Gly in saline (S.C) 2:1	Culture in N.B (S.C) + 30% Gly in Saline (S.C) 1:3	Culture in N.B (S.C) + 30% Gly in Saline (S.C) 3:1	Control Culture in N.B (S.C) + Saline (S.C) 1:1
0 Day	100	100	100	100	100
0.4	98.36	99.2	96.62	92.59	95.29
2	55.19	67.72	50.67	33.33	59.41
3	34.69	13.13	20.27	8.64	35.29
4	27.59	9.49	16.89	3.7	10.58
5	14.2	3.48	8.1	2.46	0
6	3.82	0	0	0	0
7 to 12	0	0	0	0	0

NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

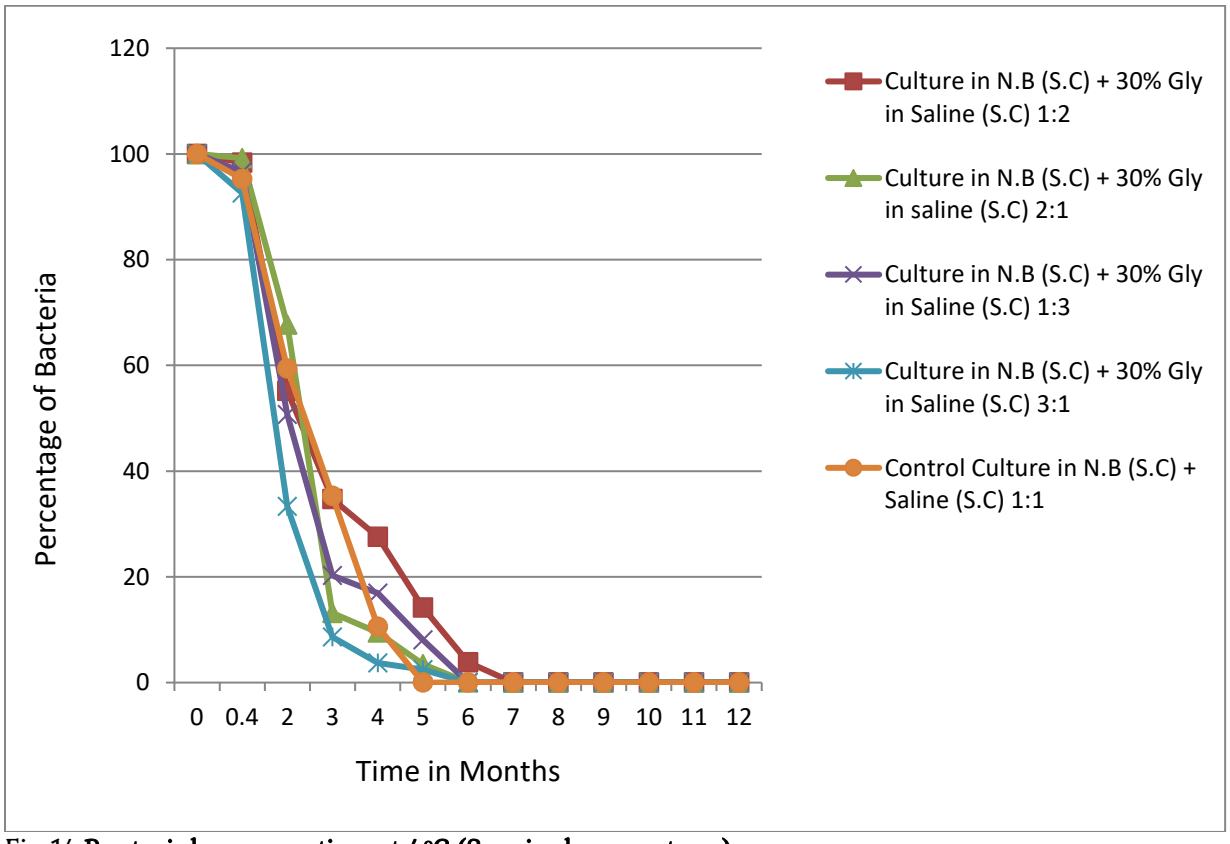

Fig 14.**Bacterial preservation at 4°C (Survival percentage)**
NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

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Table 15.**Bacterial preservation at -20°C (Survival number x 10^4)**

Time (Months)	Culture in N.B (S.C) + 30% Gly in Saline (S.C) 1:2	Culture in N.B (S.C) + 30% Gly in saline (S.C) 2:1	Culture in N.B (S.C) + 30% Gly in Saline (S.C) 1:3	Culture in N.B (S.C) + 30% Gly in Saline (S.C) 3:1	Control Culture in N.B (S.C) + Saline (S.C) 1:1
0 Day	1087	600	600	1356	382
0.4	1081	584	592	1350	375
2	732	350	380	1100	150
3	448	159	279	989	60
4	328	150	220	701	52
5	300	145	200	602	47
6	250	142	190	525	40
7	225	140	180	500	32
8	163	135	177	480	15
9	127	80	70	466	0
10	100	67	65	451	0
11	80	60	62	300	0
12	62	55	60	170	0

NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

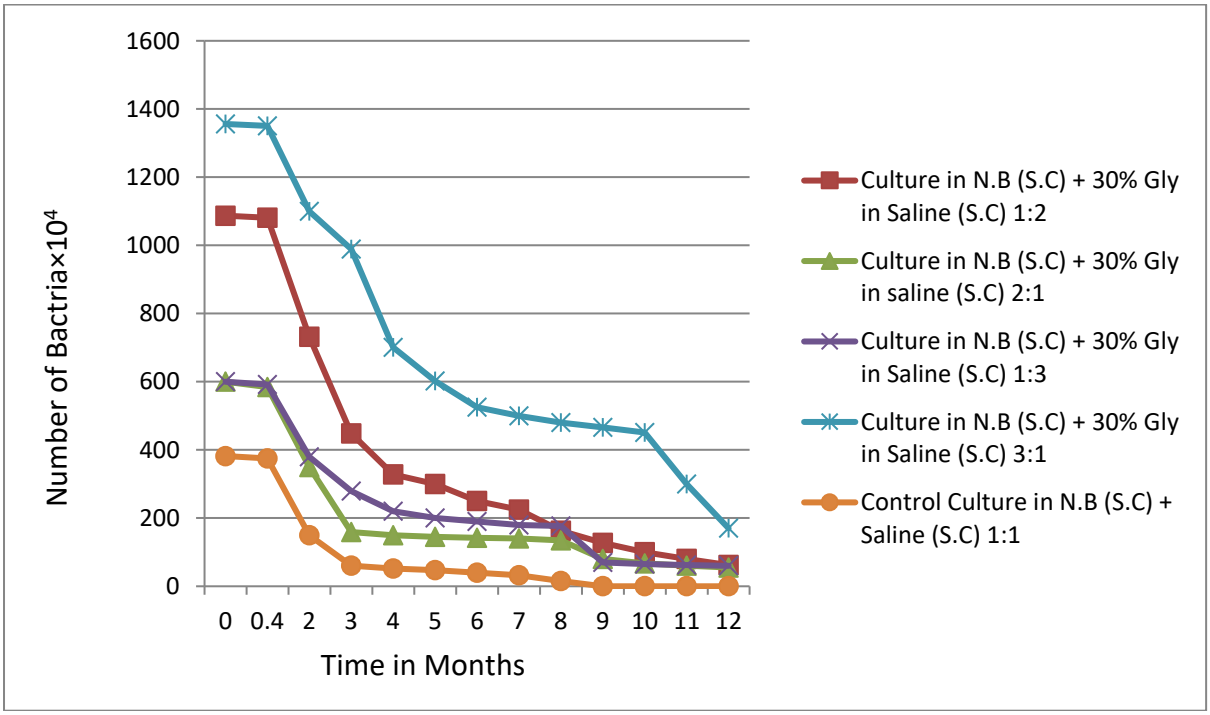

Fig. 15.**Bacterial preservation at -20°C (Survival number x 10^4)**
NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

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Table 16.**Bacterial preservation at -20°C (Survival percentage)**

Time (Months)	Culture in N.B (S.C) + 30% Gly in Saline (S.C) 1:2	Culture in N.B (S.C) + 30% Gly in saline (S.C) 2:1	Culture in N.B (S.C) + 30% Gly in Saline (S.C) 1:3	Culture in N.B (S.C) + 30% Gly in Saline (S.C) 3:1	Control Culture in N.B (S.C) + Saline (S.C) 1:1
0 Day	100	100	100	100	100
0.4	99.44	97.33	98.66	99.55	98.16
2	67.34	58.33	63.33	81.12	39.26
3	41.21	26.5	46.5	72.93	15.7
4	30.17	25	36.66	51.69	13.61
5	27.59	24.16	33.33	44.39	12.3
6	22.99	23.66	31.66	38.71	10.47
7	20.69	23.33	30	36.87	8.37
8	14.99	22.5	29.5	35.39	3.92
9	11.68	13.33	11.66	34.36	0
10	9.19	11.16	10.83	33.25	0
11	7.35	10	10.33	22.12	0
12	5.7	9.16	10	12.23	0

NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

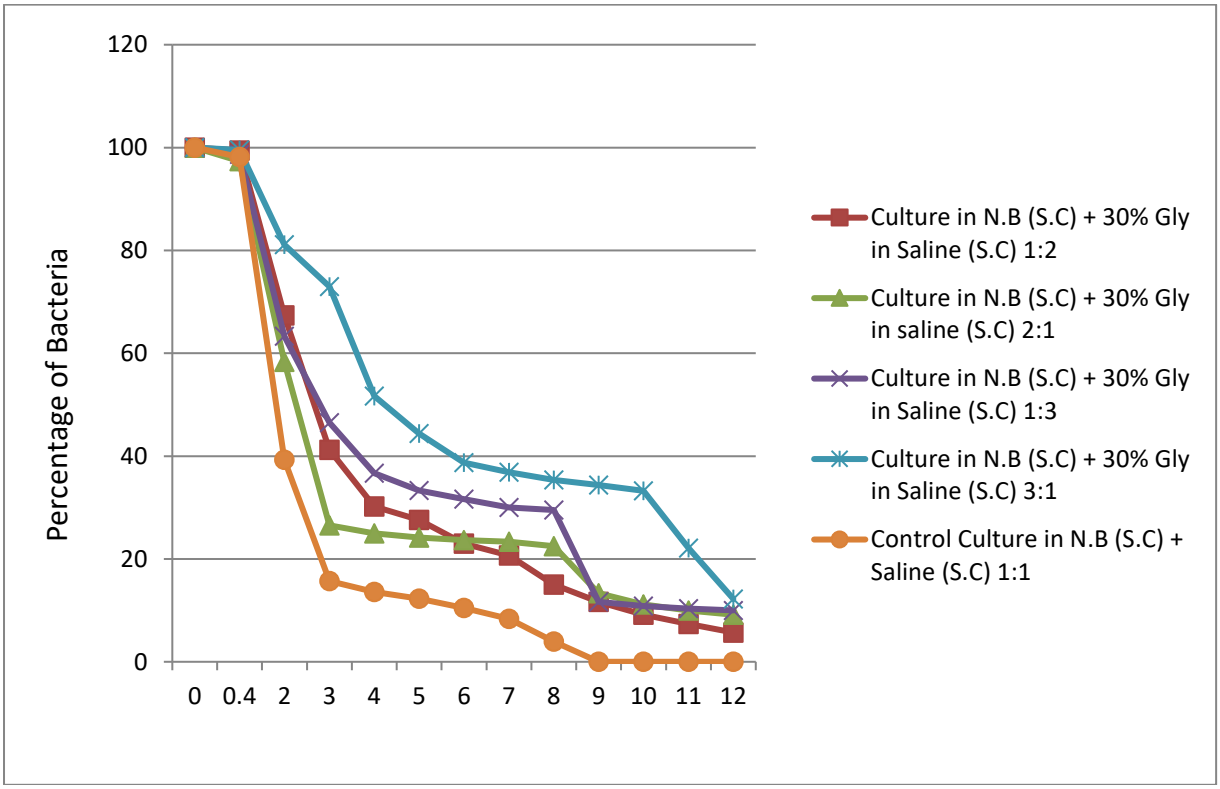

Fig 16.**Bacterial preservation at -20°C (Survival percentage)**
NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

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Table 17.**Bacterial Preservation in Various Combination of Growth Media and saline at 4° C**

Time (Months)	Culture in N.B (S.C) + 30% Gly in Saline (S.C) 1:1	Culture in N.B (S.C) + 40% Gly in Saline (S.C) 1:1	Culture in N.B (D.C) + 30% Gly in Saline (S.C) 1:1	Culture in N.B (D.C) + 40% Gly in Saline (S.C) 1:1	Culture in N.B (S.C) + 30% Gly in Saline(D.C) 1:1	Culture in N.B (D.C) + 30% Gly in Saline (D.C) 1:1	Culture in N.B (S.C) + 30% Gly in Saline (S.C) 1:2	Culture in N.B (S.C) + 30% Gly in Saline(S.C) 2:1
0 Day	950	680	3670	2990	291	312	366	632
0.4	937	672	3660	2982	284	302	360	627
2	801	501	1174	1474	184	179	202	428
3	780	476	474	400	86	67	127	83
4	430	159	450	391	70	51	101	60
5	312	21	430	342	22	10	52	22
6	25	7	219	101	6	0	14	0
7	0	0	15	7	0	0	0	0
8 to 12	0	0	0	0	0	0	0	0

NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

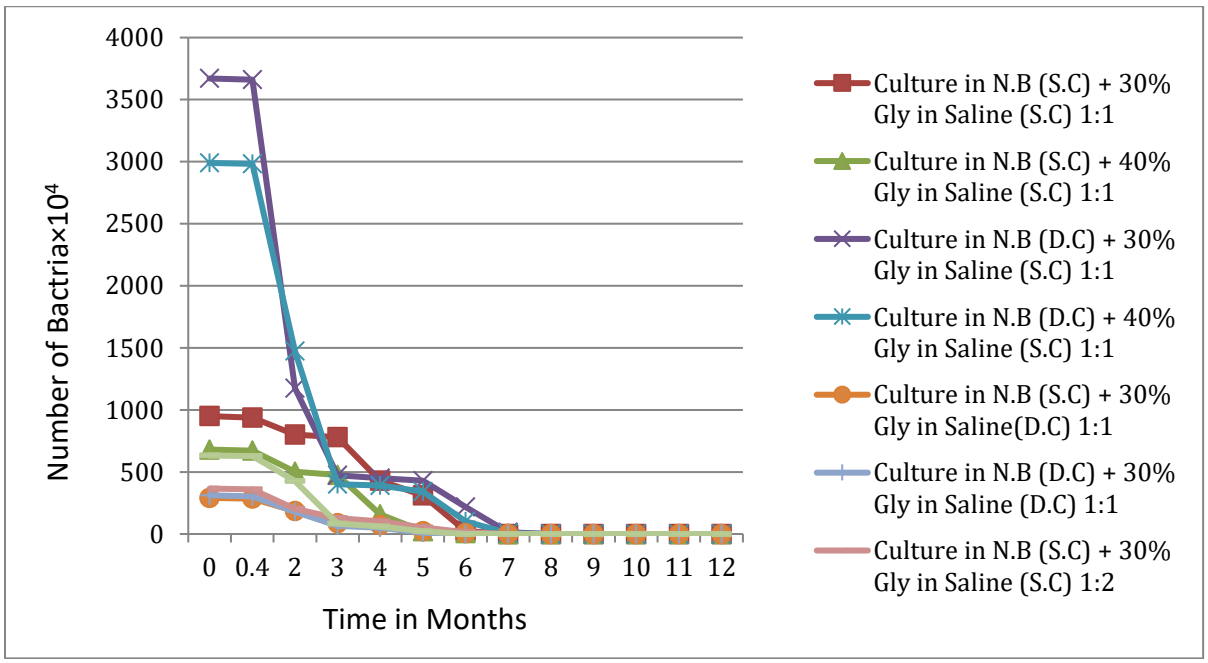

Fig 17.**Bacterial Preservation in Various Combination of Growth Media and saline at 4° C (Survival number x 10⁴)**

NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

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Table 18.:**Bacterial Preservation in Various Combination of Growth Media and saline at 4° C (Survival percentage)**

Time (Months	Culture in N.B (S.C) + 30% Gly in Saline (S.C) 1:1	Culture in N.B (S.C) + 40% Gly in Saline (S.C) 1:1	Culture in N.B (D.C) + 30% Gly in Saline (S.C) 1:1	Culture in N.B (D.C) + 40% Gly in Saline (S.C) 1:1	Culture in N.B (S.C) + 30% Gly in Saline(D.C) 1:1	Culture in N.B (D.C) + 30% Gly in Saline (D.C) 1:1	Culture in N.B (S.C) + 30% Gly in Saline (S.C) 1:2	Culture in N.B (S.C) + 30% Gly in Saline(S.C) 2:1
0 Day	100	100	100	100	100	100	100	100
0.4	98.63	98.82	99.72	99.73	97.59	96.79	98.36	99.2
2	84.31	73.67	31.98	49.29	63.23	57.37	55.19	67.72
3	82.1	70	12.91	13.73	29.33	21.47	34.69	13.13
4	45.26	23.38	12.26	13.07	24.05	16.34	27.59	9.49
5	32.84	3.08	11.71	11.43	7.56	3.2	14.2	3.48
6	2.63	1.02	5.96	3.37	2.06	0	3.82	0
7	0	0	0.4	0.23	0	0	0	0
8 to 12	0	0	0	0	0	0	0	0

NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

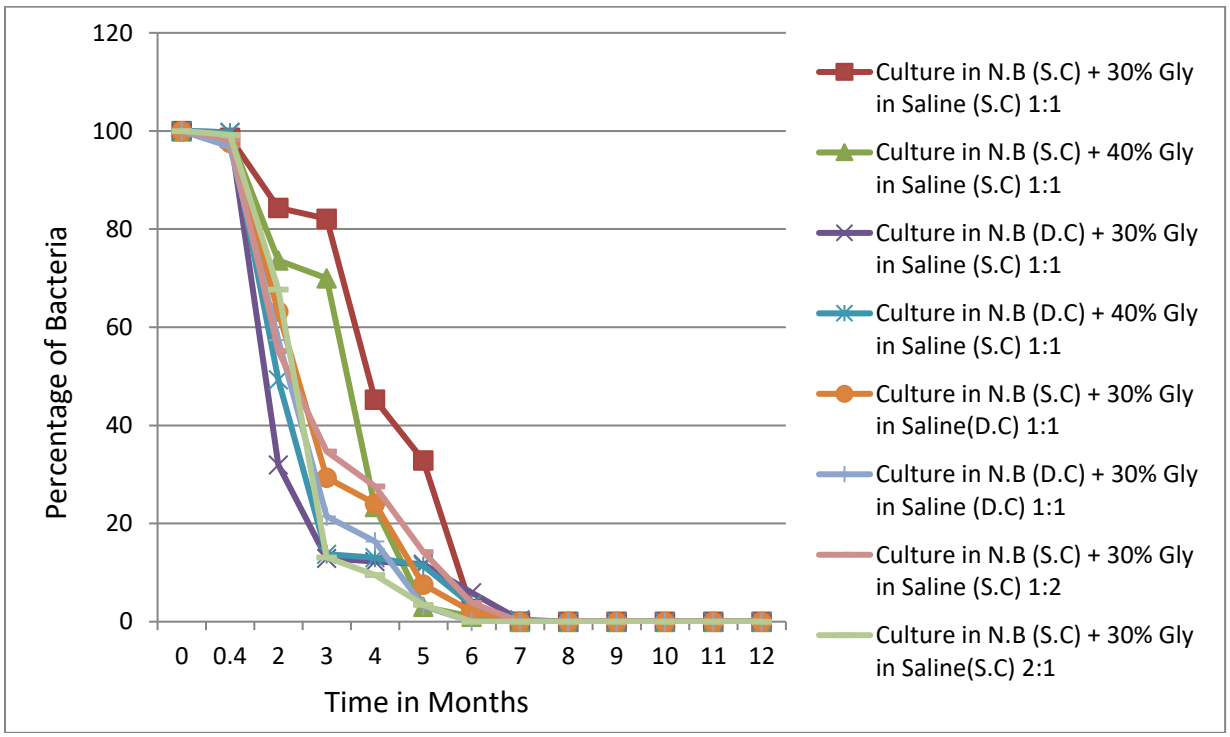

Fig 18.**Bacterial Preservation in Various Combination of Growth Media and saline at 4° C (Survival percentage)**

NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

Table 19.**Bacterial Preservation in Various Combination of Growth Media and saline at -20° C (Survival number x 10^4)**

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Time (Months	Culture in N.B (S.C) + 30% Gly in Saline (S.C) 1:1	Culture in N.B (S.C) + 40% Gly in Saline (S.C) 1:1	Culture in N.B (D.C) + 30% Gly in Saline (S.C) 1:1	Culture in N.B (D.C) + 40% Gly in Saline (S.C) 1:1	Culture in N.B (S.C) + 30% Gly in Saline(D.C) 1:1	Culture in N.B (D.C) + 30% Gly in Saline (D.C) 1:1	Culture in N.B (S.C) + 30% Gly in Saline (S.C) 1:2	Culture in N.B (S.C) + 30% Gly in Saline(S.C) 2:1
0 Day	282	639	1421	2290	1078	1174	1356	1087
0.4	276	635	1416	2287	1070	1168	1350	1081
2	147	225	1203	1500	850	830	1100	732
3	101	177	860	400	740	700	989	448
4	93	122	724	339	459	525	701	328
5	80	88	650	301	319	321	602	300
6	73	84	603	300	209	150	525	250
7	67	82	529	291	182	140	500	225
8	65	76	510	289	175	131	480	163
9	60	71	491	280	136	121	466	127
10	52	60	490	275	122	112	451	100
11	52	14	402	263	99	100	300	80
12	40	10	400	245	90	85	170	62

NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

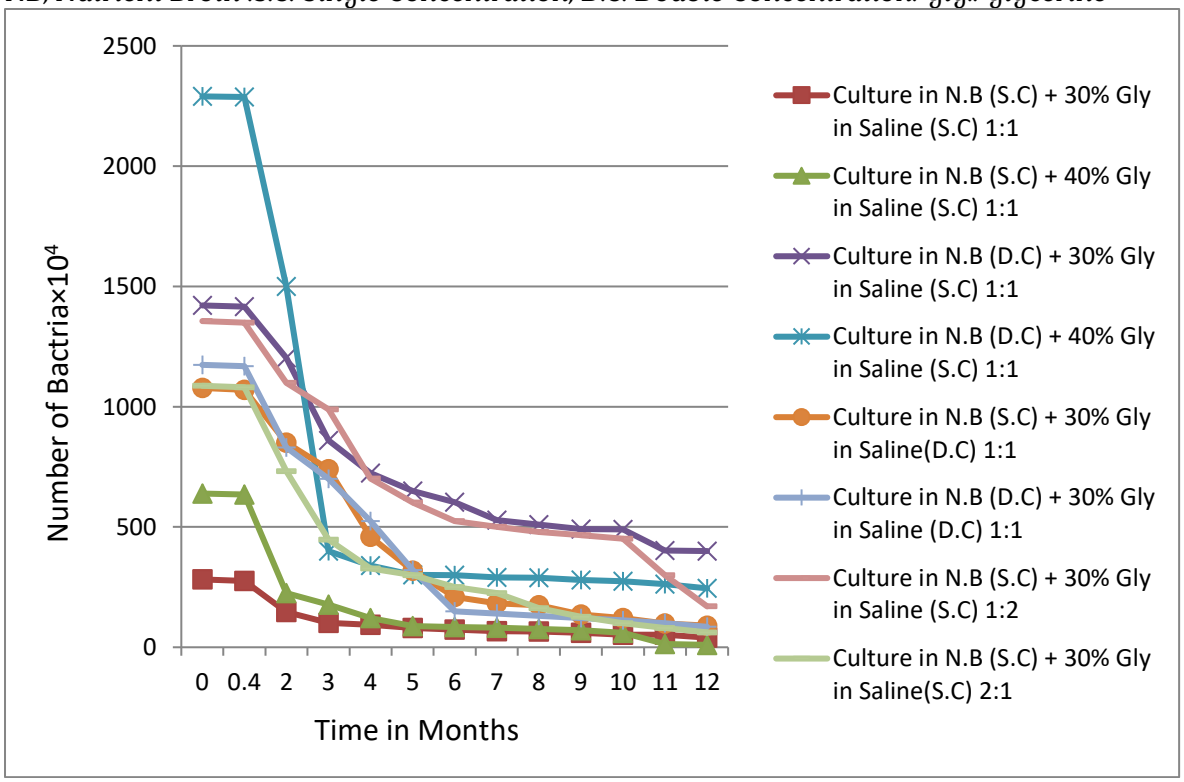

Fig 19.**Bacterial Preservation in Various Combination of Growth Media and saline at -20° C (Survival number x 10^4)**

NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

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Table 20.**Bacterial Preservation in Various Combination of Growth Media and saline at -20° C (Survival percentage)**

Time (Months	Culture in N.B (S.C) + 30% Gly in Saline (S.C) 1:1	Culture in N.B (S.C) + 40% Gly in Saline (S.C) 1:1	Culture in N.B (D.C) + 30% Gly in Saline (S.C) 1:1	Culture in N.B (D.C) + 40% Gly in Saline (S.C) 1:1	Culture in N.B (S.C) + 30% Gly in Saline(D.C) 1:1	Culture in N.B (D.C) + 30% Gly in Saline (D.C) 1:1	Culture in N.B (S.C) + 30% Gly in Saline (S.C) 1:2	Culture in N.B (S.C) + 30% Gly in Saline(S.C) 2:1
0 Day	100	100	100	100	100	100	100	100
0.4	97.87	99.37	99.64	99.86	99.25	99.48	99.55	99.44
2	52.12	35.21	84.65	65.5	78.84	70.69	81.12	67.64
3	35.81	27.69	60.52	17.46	68.64	59.62	72.93	41.21
4	32.97	19.09	50.95	14.8	42.57	44.71	51.69	30.17
5	28.36	13.77	45.74	13.14	29.59	27.34	44.39	27.59
6	25.88	13.14	42.43	13.1	19.38	12.77	38.71	22.99
7	23.75	12.83	37.22	12.7	16.88	11.923	36.87	20.69
8	23.04	11.89	35.89	12.62	16.23	11.15	35.39	14.99
9	21.27	11.11	34.55	12.22	12.61	10.3	34.36	11.68
10	18.43	9.38	34.48	12	11.31	9.54	33.25	9.19
11	18.43	2.19	28.28	11.48	9.18	8.51	22.12	7.35
12	14.18	1.56	28.14	10.69	8.34	7.24	12.35	5.7

NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

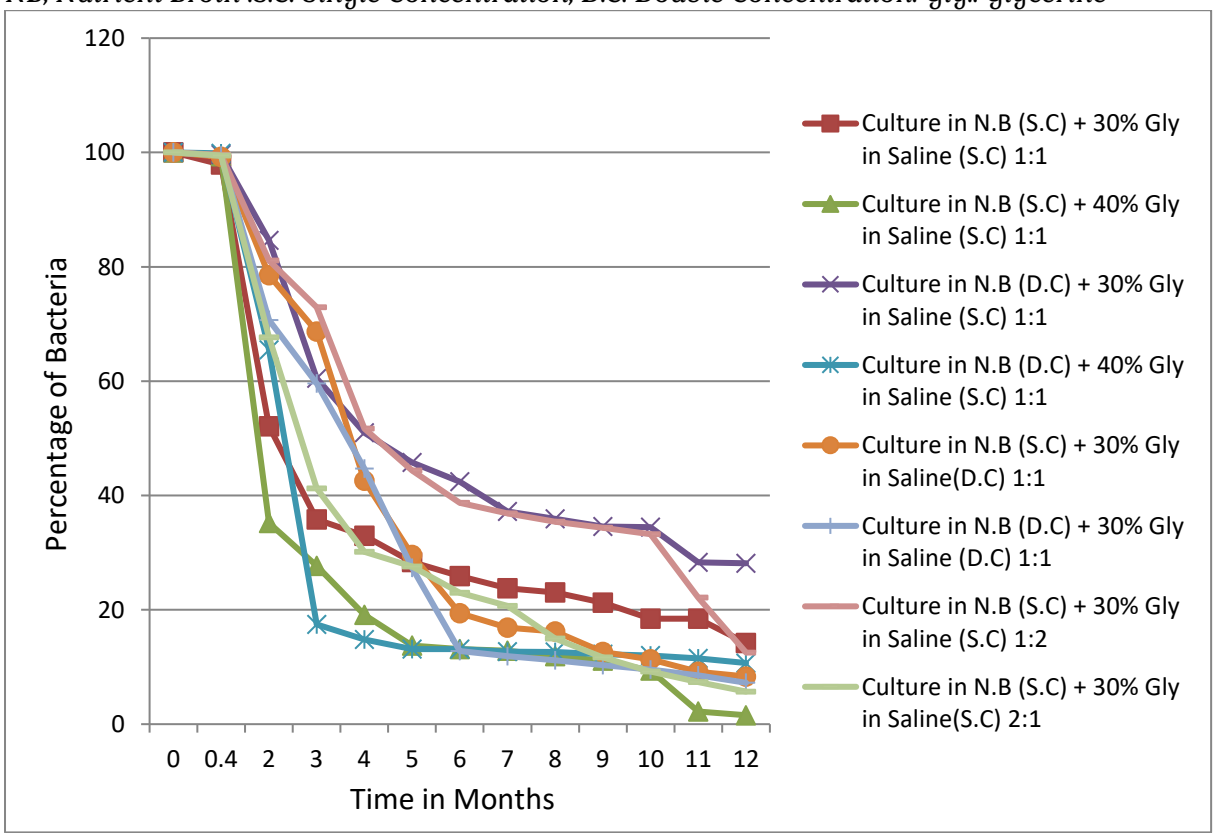

Fig 20.**Bacterial Preservation in Various Combination of Growth Media and saline at -20° C (Survival percentage)**

NB; Nutrient Broth :S.C: Single Concentration; D.C: Double Concentration: Gly.: Glycerine

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DISCUSSION:

Over the last decade, novel strategies have emerged as complementary approaches to the conventional preservation methodologies. Galacto-oligosaccharides (GOS) are polyhydroxylated carbohydrate have recently gained commercial interest as effective cryoprotectants [14].It stabilizes the membrane native structure through the replacement of water and also have a membrane protective role upon rehydration [15]

Strategies like the addition of complementary cryoprotectants to the growth media or the exposure to sublethal stress levels during the fermentation stage may lead to significant improvements in the cryotolerance ability of probiotic cells. Overall, increasing cell viability through the implementation of novel processing strategies as well as the synergistic combination of protective agents and preservation methods constitutes an important step in developing robust probiotics with attractive technological properties [16] .

Effective methodologies for culture preservation are important to ensure that the cellular properties and the biosynthetic pathways are not affected during long-term storage. In fact, such long-term genotypic and phenotypic stability will guarantee an optimum post-preservation recovery [17].

Sugars have been used for long time as preservatives in freezing and freeze-drying processes due to their ability to replace water during dehydration while maintaining the biological structures in hydrated status [18,19]. Our observation is in agreement with these researchers.

However, most of the studies involving the use of cryoprotectants in freeze-drying processes have not demonstrated enough long-term stability (>80 % survival after 1 year) of the freeze-dried bacteria at room or refrigeration temperatures [20].

Most freeze-drying cell preservation protocols include skim milk as drying

medium since it stabilizes the cell membrane constituents by creating a protective coating over the cells [21]. With the use of rapidly penetrating agents, both osmotic stress and the formation of extracellular ices are prevented and the ratio of protection is strain dependent [22].

[23]Missous *et al.* developed an artificial nucleation and temperature downshift control by adding an industrial ice nucleator protein from biological origin which led to enhanced viability of cells, when subjected tofreezing-thawing cycles .

As a thumb rule, higher microbial viability is preserved at lower storage temperature. If the storage temperature is below the freezing point, cryoprotectants are essential to reduce cell damage from the freezing process averting the deleterious influence of ice crystal formation. Glycerol, dimethylsulfoxide (DMSO), and non-permeable additives like polysaccharides are currently used as cryoprotectants in microbial cultures. Glycerol conversely acts as a membrane permeant and facilitates the vitrification process by replacing the water in the cells and making hydrogen bonds with water molecules to exert a protective effect [24]. Our work does not involve costly equipments like freeze drier, however, storage at -20°C gives sufficient long-term stability, and therefore may be opted for routine work as it withstands freezing and thawing a number of times.

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With 15% glycerol, [25]Hollander and Nell reported *E.coli remained viable* at -70°C for two months and [26]Howard in 1955 cited several bacteria ,the orgs.remained viable at -10°C for 5 months. Our findins are somewhat supported with these observations.

CONCLUSION:

1.Out of all combinations and variations in concentration of growth media and the cryoprotectant, used, it can be concluded that in those labs-

(a) Where there is no ultra freezing facility is not available, and only refrigerator (4°C) is available, the bacterial growth in nutrient broth (double concentration) mixed with equal volume of 30 to 40% glycerine (solubilized in single concentration of saline) can be used for preservation of bacteria satisfactorily upto a period of six months. Subsequently fresh stock of same media can be used for growing the organism and preserved with 30 to 40% glycerine for another six months and likewise to keep the bacteria viable, however, integrity of the organism should be checked every time to avoid mutation, occurs, if any.

(b)

(ii)Where there -20°C facility is available, bacterial growth in the above media with 30 to 40% glycerine (S.C of saline) may be used for preservation of the organism well upto a period of 12 months. Alternatively, bacterial growth in nutrient broth (single concentration)-3parts, mixed with 1 part of 30% glycerine (solubilized in single concentration of saline) also supports well upto 12 months.

2. Where, there is a regular use of the organism for research as well as in development of industrial products the organism can be utilized from the same stock. It appears that the preservation media can withstand willful freezing and thawing operation once in a month and thus upto 12 months, in addition to the similar operation due to electricity failure at least half a dozen of times in a year

3. The present invention did not require any special equipment, glass and plastic ware as required for freeze drying, foam drying, liquid nitrogen, etc, claimed by other workers for long storage. These are again expensive, cumbersome and need special care and expertise for operation and maintenance.

4. No extra floor space, manpower is required, and thus small laboratories with less investment are sufficient.

5. Cryoprotectant (glycerine), used in this invention are readily available and comparatively cheaper than other materials like dimethyl –sulphoxide(DMSO), ethylene glycol, trehalose, foetal/ new born calf serum, etc.

The resultant ideal formulations developed may to be extendible to similar other organisms.

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